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Bescheinigung Certificate Attestation

09/700462

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

98303873.8

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

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Im Auftrag

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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Libraries of oligomers labelled with different tags

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Remarks: **The title of the application as originally filed reads as follows:**
Remarques: **Reagent and method**

REAGENT AND METHOD

This invention concerns reagents of the kind which comprise a product which is built up using stepwise reactions, often chemical reactions, and associated tag moieties which track the synthetic pathway and/or the reagents used. The product will often be an oligomer and the tags define the identity and position of at least one monomer residue in the oligomer. Such reagents are useful in assay methods in which they can generate much more information than can be generated by a simple labelled analyte. Sets and libraries of such reagents can be created by combinatorial chemistry and are valuable for screening large numbers of compound e.g. for biological activity. In preferred systems according to the invention, positively charged tag groups are generated for analysis by mass spectrometry by cleavage, e.g. photocleavage of neutral molecules.

WO 95/04160 describes a reagent which comprises:

a) an analyte moiety comprising at least two analyte residues, and linked to

b) a tag moiety comprising one or more reporter groups adapted for detection by mass spectrometry, wherein a reporter group designates an analyte residue, and the reporter group at each position of the tag moiety is chosen to designate an analyte residue at a defined position of the analyte moiety. A plurality of such reagents, each comprising a different analyte moiety, provides a library of reagents which may be used in assay methods involving a target substance. Analysis of the tag moieties indicates the nature of the analyte moiety bound to the target substance.

WO 94/08051 describes a system used to make simultaneously a library of all oligomers each attached to a bead. Any individual bead made by a split and mix process carries a unique chemical

product, and this is true of each bead which goes through the same synthetic pathway. Two coupling steps are used at each point in the process: one step affects the synthon or ligand; the other alters the structure of a tag which is also carried on the bead. Tags are designed to identify the steps through which the bead has been taken.

It is an object of this invention to provide a set or library of labelled compounds which may be synthesised on a support and may be used either attached to or separated from that support.

In one aspect the invention provides a method of making a set of labelled compounds, by the use of a support and a set of labels, which method comprises the steps:

a) at least one first or intermediate step comprising dividing the support into lots, performing a different chemical reaction on each lot of the support so as either to modify that lot of the support or to couple a chemical moiety to that lot of the support, tagging a fraction of each lot of the support with a different label, and combining the said lots of the support, and

b) at least one intermediate or final step comprising dividing the support into lots, performing a different chemical reaction on each lot of the support, so as either to modify that lot of the support or to couple a chemical moiety to that lot of the support, tagging a fraction of each lot of the support with a different label, whereby each different label is linked to a chemical moiety coupled to the support in a previous step and forms with that chemical moiety a labelled compound which is separable from the support, and combining the said lots of the support.

The method uses a support which is repeatedly divided into lots which are then recombined. The support may be a massive support e.g. a flat sheet or silicon chip or microtitre plate which is divided e.g. by masking into regions for performing the different chemical reactions. The support may be a polymeric material which is soluble in some solvents and not in others, and which is separated into lots or recombined e.g. by precipitation or dissolution. Most usually the support will be particulate, e.g.

pins or fibres or preferably beads. Derivatised beads for performing combinatorial chemistry by a split and mix strategy are commercially available and can be used here. A preferred particulate support comprises beads having cleavable linkers, wherein each cleavable linker has one group for defined chemical procedures e.g. oligomer synthesis and another group for labelling. By this means it is possible at the end of the synthesis, to recover the labelled chemical products e.g. oligomers into solution.

The method of the invention involves performing at least one step a) and at least one step b), most usually at least three steps in all. Each step involves performing a reaction, generally but not necessarily a chemical reaction. An example of such a reaction might be the removal of a protective group so as to leave a primary amine or hydroxyl or carboxylic acid group. Most usually, the chemical reaction involves coupling a chemical moiety to the support. The chemical moiety will usually be an organic chemical group, for example as described in WO 94/08051. While successive chemical moieties may be attached to the support through separate linkers, more usually, successive chemical moieties will be joined to each other to form a chain extending from the support. Preferably the chemical moieties are monomer units which are built up to form oligomer chains.

In a preferred method according to the invention, the set of labelled compounds is a library of n^s labelled oligomers, where n is the number of different monomer units and s is the number of monomer units in each labelled oligomer, wherein step a) is performed once to couple a different monomer unit to each lot of the support, and step b) is performed s-1 times.

The oligomer may be for example an oligonucleotide or an oligopeptide. When the oligomer is an oligonucleotide or analogue, then n is generally 4. When the oligomer is an oligopeptide, then n is generally about 20 when only natural amino acids are used. But the principles of the invention are equally applicable to other oligomers formed from other

polymerisable monomers. The value of s is not critical, and may typically be from 2 – 100 e.g. 3 – 20 or more.

Preferably from 0.25% to 25% of each lot of the support is tagged in each step with a different label. Preferably the support has cleavable linkers, wherein each cleavable linker has at least one group for chemical reaction e.g. chemical synthesis and another group for labelling. Preferably each resulting labelled compound comprises a single label and at least one chemical moiety.

The method involves the use of a set of up to and including n x s different labels. Although the nature of the labels is not critical, it is a preferred feature of the invention that each different label be distinguishable by the analytical procedure used to detect the labels. Groups used as labels should be much more stable to acidic (or other chemical) treatment involved in oligomer synthesis compared to the protecting groups commonly used (e.g. DMT groups to provide 5' or 3'-protection in nucleotide synthons). Preferred labels are those in which a charged group, preferably a positively charged group is generated by cleavage e.g. photocleavage of a neutral molecule for analysis by mass spectrometry. Examples of such preferred labels are discussed below.

In a preferred embodiment, a split and mix strategy requires a solid support carrying cleavable linkers with three arms – one to attach to the solid support through a cleavable bond; one to initiate synthesis of a chemical product e.g. oligomer; and a third for attachment of the tags. The sites for coupling of synthon and tag monomers will optionally be protected by removable groups. The process can be illustrated by the synthesis of oligomers on a particulate solid support.

At each stage in the synthetic route, the particles of the support are first combined and mixed, and then divided into n lots, where n is the number of different monomers – 4 in the case of natural nucleotides – and each monomer is coupled to its site on one lot of the support. A unique tag representing the monomer just added and its position in the

sequence is then coupled to a fraction of the support, corresponding approximately to the number of monomers in the final oligomer (i.e. 1/s for an oligomer with s monomer units). Alternatively, a tag may be coupled to a fraction of the support before or simultaneous with, rather than after, the monomer which it represents. Partial coupling may be achieved in a number of different ways. For example, (i) a protecting group on the site may be partially removed; (ii) the coupling may be taken to a fraction of completion; (iii) a fraction of the support may be removed and coupling taken to completion before the fraction is returned to the pool. As the coupling steps proceed, the oligomer is extended one unit at a time, and the tags are added one at a time. The end result is a mixture of molecules on each particle; each molecule will carry the same sequence of monomers in the oligomer, but a fraction, 1/s for s-mers, will carry the tag added at any of the s coupling steps.

An example of this embodiment is shown in Figure 1 of the accompanying drawings. At A, is illustrated a solid support in the form of a bead derivatised with cleavable linkers each having two arms. At B, one of the two arms of each linker has been reacted with a trityl group carrying a succinimidyl substituent. At C, the other branch of each linker has been reacted with a nucleotide residue shown as G; and one portion of the NHS groups has been substituted by a label R_1. At D, oligonucleotide synthesis has continued by formation of dimer chains GT; and a second portion of the NHS groups has been substituted by a second label R_2. At E, oligonucleotide synthesis has continued by formation of chains GTT; and a third portion of the NHS groups has been substituted by a label R_3. At F, ammonolysis of the beads has given rise to a pool of oligonucleotides of the same sequence, in which each one is attached to a different tag. At G, photolysis has detached three derivatised trityl groups for analysis by mass spectrometry. The split and mix approach ensures that all the oligonucleotides attached to any bead have the same s-mer sequence; and that the bead also carries a total of s different labels, each of which

indicates the position and identity of one monomer residue of the oligomer.

An alternative way of partial coupling is to cap the extension of a fraction of the chemical compounds e.g. oligomers with a stable tag group at each extension step. For example, in the case of oligonucleotide synthesis, the coupling agents could include a small proportion of a phosphoramidite protected by one of the stable trityl groups described below as mass tags. Elongation will produce a major proportion with the desired base and a small fraction with a corresponding tag marking the nature and position of the base.

An example of this embodiment is illustrated in Figure 2 of the accompanying drawings. Oligonucleotide synthesis is performed on derivatised beads A, the first, second and third stages of this synthesis being shown as B, C and D. Each of four phosphoramidite reagents contains a small fraction depending on the length of the oligomer, preferably less than 1/s, of a capping phosphoramidite bearing a very acid-stable NHS-substituted trityl group. After each stage of synthesis, all incorporated NHS groups are reacted with an amine thereby attaching a label. For synthesis of longer oligonucleotides o-methyl phosphoramidite could be used to withstand repetitive amination reactions. The three different labels used in B, C and D are shown in Figure 2 as R_1, R_2 and R_3. At the end of synthesis, the oligonucleotides are deprotected by treatment with ammonia, but remain attached to the beads. Thus each bead carries a plurality of s-mer oligomers of identical sequence, together with a total of s different substituted trityl labels each of which indicates the identity and position of a monomer unit of the oligomer. The beads are used in an assay procedure. Thereafter photolysis of a bead generates charged substituted trityl moieties for detection by mass spectroscopy. Alternatively the labelled oligonucleotide can be released into solution.

In another aspect, this invention provides a set of labelled compounds wherein a molecule of a compound of the set is tagged with a single label which identifies the nature and/or the position of a component

of that molecule, and different molecules of the same compound are tagged with different labels. The set of labelled compounds may be releasably attached to a solid support e.g. beads; or may be mixed together in solution.

In one preferred embodiment (e.g. that illustrated in Figure 1) the set of labelled compounds consists of s different labelled oligomers, wherein each different labelled oligomer has the same sequence of s monomer residues and is linked to a different label which indicates the identity and position of a single monomer residue of the oligomer. In another preferred embodiment the set of labelled compounds consists of s different labelled oligomers, with different oligomer molecules having the same sequence but different lengths up to s monomer residues, and wherein each oligomer carries a label which identifies the nature and position of a monomer residue of the oligomer molecule.

Also envisaged according to the invention is a library consisting of a plurality of the sets of the labelled compounds as herein defined, e.g. a library of n^s labelled oligomers, where n is the number of different monomer units and s is the number of monomer units in each labelled oligomer.

In another aspect (e.g. as illustrated in Figure 2) the invention provides a reagent comprising a solid support which carries on its surface molecules of an oligomer, with different oligomer molecules having the same sequence but different lengths, wherein one end of an oligomer molecule is linked to the solid support and the other end carries a label which identifies the nature and position of a monomer unit of the oligomer molecule. A library consists of a plurality of the said reagents, in which the solid supports are preferably beads.

Preferred features of the labels used herein are:

- They should be attached by linkages which are stable to the chemical procedures used in the preparative method and those used to detach the resulting chemical compound e.g. oligomer from a solid support.

The trityl residues described below are stable throughout the procedures used to synthesise oligonucleotides.

- They should have properties which allow up to n x s labels to be distinguished by the analytical procedure used to detect them, as each chemical moiety or reaction is tagged uniquely. In an example below, it is shown how all 262144 nonanucleotides can be coded uniquely using 36 different tag monomers. This number is readily achieved using the trityl derivatives described below. Alternatively, but less preferably, the same number of 9-mers could be coded for by 18 binary tags or even by a unique combination of 9 tags as described in WO 94/08051.
- On cleavage, e.g. photocleavage, from the parent molecule, they should generate stable species, either neutral molecules or preferably charged ions, for analysis by mass spectrometry. Mass spectrometry is a preferred method of analysis, allowing for the simultaneous detection of hundreds of labels. This property, of generating a preferably charged group by photocleavage of a neutral molecule, ensures that the ions are brought into the vapour phase without the need for added matrix. Therefore it is not necessary to search for "hot spots" as is the case when matrix is added. Not having matrix present also allows for further biochemical processes e.g. oligonucleotide ligation.

Bearing in mind these criteria, preferred labels according to the invention are groups of the formula $R^1R^2R^3X-$ or R^1R^2N-, where X is C or S and R^1, R^2 and R^3 are the same or different and each is a monocyclic or fused ring aromatic group that is substituted or unsubstituted. Preferably X is C, in which case these are groups of the trityl (triphenylmethyl) family. Other possible labels include troponium and those discussed in WO 97/27331. Trityl groups have the desirable property that they are readily cleaved by illumination with a laser in a mass spectrometer. Sensitivity of detection of trityl groups is high because of the stability of the positively charged carbonium ion. This sensitivity gives rise to a number of advantages, e.g. there are enough trityl groups in a molecular monolayer

such as results if trityl labelled molecules are tethered covalently to a surface.

Preferably at least one of R^1, R^2 and R^3 carries a substituent selected from C_1-C_{20} alkoxy or hydrocarbyl either unsubstituted or substituted by carboxylic acid, sulphonic acid, nitro, cyano, hydroxyl, thiol, primary, secondary or tertiary amino, primary, secondary or tertiary amido, anhydride, carbonyl halide or active ester.

Preferably each of R^1, R^2 and R^3 is aryl, more preferably phenyl. While substituents may be present at any point in the aromatic (e.g. phenyl) ring, para-substituents are convenient and are preferred. The substituents may be present to confer desired physical or chemical properties on the trityl (or other) group. For example, electron withdrawing groups at ortho or para positions increase the stability of trityl groups to acid hydrolysis. Substituents may be present to alter the formula weight of the trityl (or other) group, so as to enable easy detection and discrimination by mass spectrometry. Non-radioactive isotopic substituents are suitable for this purpose, e.g. small alkyl groups containing 1, 2 or 3 deuterium atoms. Preferred substituents are amine or amide groups. There is a considerable number of amines having different molecular weights that are commercially available and that can be used to provide substituted trityl groups having distinctive formula weights, see for example Table 1 below.

The masses of the majority of commercially available amines lie in the range of 50 – 250 Da. For some applications it would be desirable to have up to a few hundred mass-tags. The resolution of the tags in TOF-mass spectrometry was found to be satisfactory with at least 4 Da difference between the masses of tags. Therefore, the above range of amines can only yield about 50 different tags. To increase this amount using the same pool of amines, it is possible to incorporate two or four or even more amide substituents per trityl group, and this is illustrated in the experimental section below.

The principle of the system is illustrated in Figure 3 of the

accompanying drawings. At A, an oligonucleotide has been synthesised on a CPG support. At B, a 5'-hydroxyl group of the oligonucleotide has been replaced by an NHS-substituted trityl group. At C, an amide group NHR has been introduced, in which R is chosen to have a characteristic formula weight. At D, the labelled oligonucleotide has been released into solution for use in an assay procedure. At E, the NHR-substituted trityl group has been volatilised by photolysis and has been detected by mass spectrometry.

The above mass spectrometry labels are useful in a variety of other biochemical methods and manipulations. Thus according to another aspect, the invention provides a nucleic acid determination method, which method comprises providing a labelled oligonucleotide or nucleic acid, and removing the label by photocleavage to give a charged species which is subjected to mass spectrometry. Preferably nucleic acid sequencing is performed by the use of a labelled primer and/or labelled chain extending nucleotides and/or labelled chain terminating nucleotide analogues, wherein the label is as described above.

In another aspect, the invention provides an assay method in which a labelled probe is partitioned into two fractions of which one is determined, the probe comprising a ligand joined to a label by a link which is photocleavable to give a charged species for analysis by mass spectrometry. The invention also includes a library of probes, each comprising a ligand joined to a label by a link which is photocleavable to give a charged species for analysis by mass spectrometry, wherein each different probe has a different label. Preferably the labels are as described above.

Certain of the labels are envisaged as new compounds *per se* according to the invention. These are compounds of the formula $R^1R^2R^3XY$ or R^1R^2NY; where X is C or S preferably C; Y is a leaving group for reaction with a nucleophilic species, e.g. a halide, tosylate, thiol, alcohol or amine group; and R^1, R^2 and R^3 are as defined above, with the

proviso that R^1, R^2 and R^3 together carry at least two amide groups and/or at least two reactive groups for coupling e.g. N-hydroxysuccinimide ester groups.

EXPERIMENTAL

A series of compounds has been made with different substituents at the phenyl rings of the core trityl structure. Some of these compounds are believed to be new and form additional aspects of this invention. The chemistry involved is illustrated in Figure 4 of the accompanying drawings. The compounds and their properties are as follows.

Example 1

N-succinimidyl-4-[bis-(4-methoxyphenyl)-chloromethyl]-benzoate (1) was synthesised according to the reported procedures (1,2).

N-succinimidyl-4-[(4-methoxydiphenyl)-chloromethyl]-benzoate (2) was synthesised according to the reported procedures (1,2), but 4-methoxybenzophenone was used in the Grignard synthesis instead of 4, 4'-dimethoxybenzophenone. ^{1}H-NMR ($CDCl_3$, δ, md): 7.95-6.8 (m, 13H, arom.), 3.8 (s, 3H, OCH_3), 2.88 (s, 4H, NHS). Mass-spectrum, TOF (no matrix): (MI + H^+).

N-succinimidy-4-[bis-(phenyl)-chloromethyl]-benzoate (3) was synthesised according to the reported procedures (1,2), but benzophenone was used in the Grignard synthesis instead of 4, 4'-dimethoxybenzophenone. ^{1}H-NMR ($CDCl_3$. δ, md): 8.0-6.8 (m, 14H, arom.), 2.88 (s, 4H, NHS). Mass-spectrum, TOF (no matrix): (MI + H^+).

The formation of the Grignard reagent from 2-(4-bromophenyl)-4,4-dimethyl-1,3-oxazoline is rather unreliable and irreproducible, even using RED-Al®(Aldrich) as an activator. To take advantage of commercially available Grignard reagents (Aldrich), the

inventors synthesised oxazolyl-protected 4-carboxybenzophenone (**4**) starting from 4-carboxy benzophenone. Following the Grignard reaction, subsequent steps were similar to those used for compounds **1-3**.

2-(4-benzophenyl)-4,4-dimethyl-1,3-oxazoline (4). 4-benzoylbenzoic acid (50g, mmol) was refluxed in 300 ml of thionyl chloride for 3h, evaporated (the product crystallises from the oil) and then evaporated with toluene (2 x 30 ml). The residue was dissolved in 254 ml of dry methylene chloride. To this ice-cooled solution, 46g (2.5 eqv) of 2-amino-2-methylpropanol-1 in 150 ml of dry methylene chloride was added dropwise for 2h. The solution was stirred overnight at rt, and the precipitate was washed several times with methylene chloride. Combined fractions were evaporated, carefully dissolved in 350 ml of thionyl chloride and refluxed for 4h. The reaction mixture was evaporated to 1/3, poured into 2L of dry ether and kept overnight at 4°C. The precipitate of hydrochloride was dissolved in 1L of water at 10°C, and 300ml of 5M KOH was added to it with stirring. The mixture was extracted with chloroform (3 x 350ml), organic phase dried over $CaCl_2$ and evaporated. The crystalline product was obtained from toluene. 42g (75%) white crystalline solid, mp 81°C. ^{1}H-NMR ($CDCl_3$. δ, md): 8.2-7.2 (m, 9H, arom.), 4.18 (s, 2H, CH_2), 1.45 (s, 6H, CH_3). Mass-spectrum, MALDI-TOF: 279.091 (MI), 302.085 (MI + Na^+), 319.656 (MI + K^+).

The masses of the majority of commercially available amines lay in the range of 50-250 Da. For some applications it would be desirable to have up to a few hundred mass-tags. The resolution of the tags in TOF mass-spectrometry was found to be satisfactory with at least 4 Da difference between the masses of tags. Therefore, the above range of amines can only yield about 50 different tags. To increase this amount using the same pool of amines, the inventors synthesised another trityl-based compound with two activated carboxyl groups (**6**), which upon reaction with amine would form two amide bonds thus giving other series of

mass-tags as compared, for example, to **(2)**. Additional increase of mass can be achieved by attaching even more amines to trityl core by using, for example, **(7)**.

4,4'-[bis-(2-(4,4-dimethyl-1,3-oxazolyl))]-4''-methoxytritanol (5). To 1.5g of magnesium turnings activated with iodine 15.34g (mmol) of bromophenyl oxazoline in 150ml of dry THF and a droplet of RED-A1® were added with stirring and the mixture was refluxed for 3h, cooled to rt and 4.64g (mmol) of methyl 4-methoxybenzoate in 40ml of dry THF was added dropwise. The mixture was gently refluxed for 6h, cooled to rt and 10 ml of water was added with stirring. Organic phase was carefully decanted and residue washed several times with small portions of THF. Combined organic fractions were evaporated and purified (flash-chromatography) to give 11.4g (84%) of light yellow solid. Mass-spectrum, MALDI-TOF: 467.545 (MI - OH), 484.869 (MI). ^{1}H-NMR ($CDCl_3$, δ, md): 7.95-6.75 (m, 12H, arom.), 4.12 (s, 4H, CH_2), 3.78 (s, 3H, OCH_3), 1.37 (s, 12H, CH_3).

***4,4'-[bis-(2-(succinimidylcarboxy)]-4''-methoxytrityl chloride* (6).** The solution of **5** (10g, mmol) in 250ml of 80% acetic acid was kept at 72°C for 48h, evaporated and then evaporated with water (2 x 50 ml). The product was dissolved in 75ml of 50% EtOH/water, refluxed for 3h and evaporated to 1/3. The mixture was then dissolved in 100ml of water and acidified with 3M HCl to pH 1-2. The precipitate was dissolved in chloroform, dried (Na_2SO_4) and evaporated to dryness and additionally dried *in vacuo* overnight. Dicarboxylic acid obtained was dissolved in 100ml of dry THF. 8.5g (mmol) of N-hydroxysuccinimide was added and the mixture was cooled to 0°C. Dicyclohexylcarbodiimide (8.5g, mmol) in 20ml of dry THF was added dropwise with stirring. The reaction mixture was stirred 1h at 0°C and overnight at rt. Dicyclohexylurea was filtered off and organic phase was evaporated to dryness and purified (flash-chromatography) to give 8.5g (%) of white yellow-white solid. Mass-spectrum, MALDI-TOF:

554.703 (MI + OH), 570.794 (MI). ^{1}H-NMR ($CDCl_3$ δ, md): 8.2-6.75 (m, 12H, arom.), 3.81 (s, 3H, OCH3), 2.9 (s, 8H, CH2). This compound was converted into corresponding trityl chloride by refluxing in AcCl/toluene for 3h. The reaction mixture was then evaporated to 1/3. 2/3 of volume of toluene was added, the mixture was again evaporated to 1/3 and used without further purification.

To evaluate these modified trityl blocks as a mass-tags, compounds **1** and **2** were used to synthesise 5'-protected thymidines as described in (3). 0.1M solutions of these nucleosides in THF were reacted with 0.1M solutions of amines in THF or dioxane, by mixing 10 μl of each. The mixtures were analysed on MALDI-TOF Voyager Elite Biospectrometry research station, PerSeptive Biosystems, without using any matrix, to prevent formation of molecular ions. Typical results are presented in the table.

All synthesised trityl blocks were tested for acid-lability by treatment with 2-5% TsOH or $HClO_4$ of corresponding 5'-thymidylates and TLC-analysis of the products after quenching with sat. sodium bicarbonate. As expected, there was about one order of magnitude difference in stability between DMTr, MMTr and Tr. Corresponding NHS-derivatives were about twice as stable, i.e. the stability was: DMTr < DMTr(NHS) < MMTr < MMTr(NHS) < Tr < Tr(NHS).

REFERENCES

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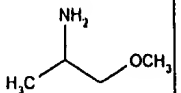
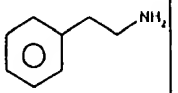
3. Gait, M.J. (1984) *Oligonucleotide Synthesis. A Practical Approach.* IRL Press, Oxford, UK.

Table 1

Amine (MW) / Trityl	NH_3 (17)	H_3CNH_2 (31.06)	$H_3CCH_2NH_2$ (45.09)	$H_3C(CH_2)_2NH_2$ (59.11)	$H_3C(CH_2)_3NH_2$ (73.14)	$H_3C(CH_2)_4NH_2$ (87.17)	 (89.14)	$H_3C(CH_2)_5NH_2$ (101.19)	$H_3C(CH_2)_6NH_2$ (115.22)	 (121.18)	$H_3C(CH_2)_7NH_2$ (129.25)
DMTr(NHS)	346.082	359.276	373.188	388.308	402.18	415.179	416.998	428.777	442..939	449.075	456.326
MMTr(NHS)	315.448	329.109	342.639	357.249	371.945	384.92	386.585	399.53	412.198	418.943	426.521

Example 2

Thiolated oligonucleotides have previously been used to immobilise PCR products onto gold monolayers (e.g. Hegner *et al.*). The attachment is due to bonding between the gold and the thiol group. Using this chemical reaction it is possible to immobilise any gene, or region of any gene, onto the gold plated surface of a mass spectrometer target plate, via a thiol linkage.

This example illustrates the immobilisation of PCR products to the gold surface of mass spectrometer target plates; hybridisation and ligation of pairs of oligonucleotides, one of which defines the locus and the other a putative allele; the allele is characterised by the detection of a trityl tag(s) by mass spectrometer. The example given uses M13mp18 ssDNA as a putative target region.

PCR and immobilisation (Figure 5)

A 225 base pair PCR product was amplified from M13mp18 ssDNA using two oligonucleotide primers;
A1 5'ACTGGCCGTCGTTTTAC3'- ; B1 5'AAGGGCGATCGGTGCGG 3'-.
A1 was synthesised with the addition of a 17 atom linker molecule, and a thiol group, to the 5' end using conventional phosphoramidite chemistry (see figure). The thiol group was activated with a 200 fold excess of DTT, and 5ng of product was spotted on to the gold target plate. Incubation in 100% humidity overnight was sufficient to immobilise the PCR product. Excess template was removed by flooding the plate with 10mM Tris-HCl (pH 7.5).

Hybridisation and ligation (Figure 6)

The oligonucleotide defining, the locus,
C1 - 5'GTAAAACGACGGCCAGT3' was synthesised with a phosphate group coupled to the 5' end. Two putative allele defining oligonucleotides were synthesised, D1 and D2- 5'CACGACGTT3' differing only in their 5'

terminus where D1 was tagged with dimethoxytrityl and D2 with monomethoxytrityl. Both oligonucleotides were synthesised using conventional phosphoramidite chemistry and were fully complementary to the PCR amplified product.

Ligation and hybridisation was at 46°C (Housby and Southern, 1998) overnight in 100% humidity, and under saturating concentrations of oligonucleotides C1 and D1 and/or D2. For ligation, a thermostable DNA ligase, *Tth,* was used because of its high temperature optimum for ligation and high degree of discrimination for the 3' end of substrate oligonucleotide. Residual unligated oligonucleotides were removed by washing (see figure).

Mass spectrometer analysis

The mass spectrum of monomethoxytrityl (MMT, mass 272) clearly demonstrated cleavage of MMT from the ligated product. No matrix was used to assist ionisation. Control samples showed no detectable peaks at 272.

The same experiment using dimethoxytrityl as the tagged oligonucleotide demonstrates that DMT also "flies" well and has a peak at 303 mass units.

Several oligonucleotides have been synthesised and coupled with aminated DMT and MMT derivatives as described (see table). A selection of DMT tagged oligonucleotides have been mixed together and analysed by mass spectrometry (see figure 7.)

<u>References</u>

Hegner, M., Wagner, P., and Semenza, G. (1993) FEBS 336: 452-456.

Housby, J.N, and Southern, E.M. (1998) *Nucl. Acids. Res.* Submitted.

CLAIMS

1. A method of making a set of labelled compounds, by the use of a support and a set of labels, which method comprises the steps:

a) at least one first or intermediate step comprising dividing the support into lots, performing a different chemical reaction on each lot of the support so as either to modify that lot of the support or to couple a chemical moiety to that lot of the support, tagging a fraction of each lot of the support with a different label, and combining the said lots of the support, and

b) at least one intermediate or final step comprising dividing the support into lots, performing a different chemical reaction on each lot of the support, so as either to modify that lot of the support or to couple a chemical moiety to that lot of the support, tagging a fraction of each lot of the support with a different label, whereby each different label is linked to a chemical moiety coupled to the support in a previous step and forms with that chemical moiety a labelled compound which is separable from the support, and combining the said lots of the support.

2. The method of claim 1, wherein the support is a particulate solid support.

3. The method of claim 1 or claim 2, wherein step b) is performed to couple the chemical moiety to a chemical moiety previously coupled to the support.

4. The method of claim 3, wherein the chemical moieties are monomer units and the labelled compounds are oligomers.

5. The method of claim 4, wherein the set of labelled compounds is a library of n^s oligomers, where n is the number of different monomer units and s is the number of monomer units in each labelled oligomer, wherein step a) is performed once to couple a different monomer unit to each lot of the support, and step b) is performed s-1 times.

6. The method of claim 5, wherein the set of labels contains n x s different labels.

7. The method of any one of claims 1 to 6, wherein each labelled compound comprises a single label and at least one chemical moiety.

8. The method of any one of claims 1 to 7, wherein the support is treated to release the said labelled compounds into solution.

9. The method of any one of claims 1 to 8, wherein from 0.25% to 25% of each lot of the support is tagged in each step with a different label.

10. The method of any one of claims 1 to 9, wherein the support has cleavable linkers, wherein each cleavable linker has at least one group for chemical synthesis and another group for labelling.

11. The method of any one of claims 1 to 10, wherein the label is joined by a link that is photocleavable to give a charged species for mass spectrometry.

12. The method of any one of claims 1 to 11, wherein each label is a group of formula $R^1R^2R^3X-$ or R^1R^2N-, where X is C or S, and R^1, R^2 and R^3 are the same or different and each is a monocyclic or fused ring aromatic group that is substituted or unsubstituted.

13. The method of claim 12, wherein at least one of R^1, R^2 and R^3 carries a substituent selected from C_1-C_{20} alkoxy or hydrocarbyl either unsubstituted or substituted by carboxylic acid, sulphonic acid, nitro, cyano, hydroxyl, thiol, primary, secondary or tertiary amino, primary, secondary or tertiary amido, anhydride, carbonyl halide or active ester.

14. The method of any one of claims 1 to 13, wherein the labelled compounds are labelled oligonucleotides.

15. A set of labelled compounds wherein a molecule of a compound of the set is tagged with a single label which identifies the nature and/or the position of a component of that molecule, and different molecules of the same compound are tagged with different labels.

16. The set of claim 15, wherein the labelled compounds are releasably attached to a solid support.

17. The set of claim 16, wherein the solid support is particulate.

18. The set of claim 15, wherein the labelled compounds are mixed together in solution.

19. The set of any one of claims 15 to 18, wherein the set consists of s different labelled oligomers, wherein each different labelled oligomer has the same sequence of s monomer residues and is linked to a different label which indicates the identity and position of a single monomer residue of the oligomer.

20. The set of any one of claims 15 to 18, wherein the set consists of s different labelled oligomers, with different oligomer molecules having the same sequence but different lengths up to s monomer residues, and wherein each oligomer carries a label which identifies the nature and position of a monomer residue of the oligomer molecule.

21. The set of any one of claims 15 to 20, wherein the label is joined by a link that is photocleavable to give a charged species for mass spectrometry.

22. The set of any one of claims 15 to 21, wherein each label is a group of formula $R^1R^2R^3X-$ or R^1R^2N-, where X is C or S, and R^1, R^2 and R^3 are the same or different and each is a monocyclic or fused ring aromatic group that is substituted or unsubstituted.

23. The set of claim 22, wherein at least one of R^1, R^2 and R^3 carries a substituent selected from C_1-C_{20} alkoxy or hydrocarbyl either unsubstituted or substituted by carboxylic acid, sulphonic acid, nitro, cyano, hydroxyl, thiol, primary, secondary or tertiary amino, primary, secondary or tertiary amido, anhydride, carbonyl halide or active ester.

24. The set of any one of claims 15 to 23, wherein the labelled compounds are labelled oligonucleotides.

25. A library consisting of a plurality of the sets of any one of claims 19 to 24.

26. A reagent comprising a solid support which carries on its surface molecules of an oligomer, with different oligomer molecules having the same sequence but different lengths, wherein one end of an oligomer molecule is linked to the solid support and the other end carries a label which identifies the nature and position of a monomer unit of the oligomer molecule.

27. The reagent as claimed in claim 26, wherein the solid support is a bead.

28. The reagent as claimed in claim 26 or claim 27, wherein the label is joined by a link that is photocleavable to give a charged species for mass spectrometry.

29. The reagent of any one of claims 26 to 28, wherein each label is a group of formula $R^1R^2R^3X-$ or R^1R^2N-, where X is C or S, and R^1, R^2 and R^3 are the same or different and each is a monocyclic or fused ring aromatic group that is substituted or unsubstituted.

30. The reagent of any one of claims 26 to 29, wherein at least one of R^1, R^2 and R^3 carries a substituent selected from C_1-C_{20} alkoxy or hydrocarbyl either unsubstituted or substituted by carboxylic acid, sulphonic acid, nitro, cyano, hydroxyl, thiol, primary, secondary or tertiary amino, primary, secondary or tertiary amido, anhydride, carbonyl halide or active ester.

31. The reagent of any one of claims 26 to 30, wherein the oligomers are oligonucleotides.

32. A library consisting of a plurality of the reagents of any one of claims 26 to 31.

33. A method, which method comprises providing a labelled oligonucleotide or nucleic acid, and removing the label by photocleavage to give a charged species which is subjected to mass spectrometry.

34. The method of claim 33, wherein nucleic acid sequencing is performed by the use of a labelled primer and/or a labelled hybridisation probe and/or labelled chain extending nucleotides and/or labelled chain

terminating nucleotide analogues, wherein the label is one which is removed by photocleavage to give a charged species which is subjected to mass spectrometry.

35. An assay method in which a labelled probe is partitioned into two fractions one of which is determined, the probe comprising a ligand joined to a label by a link which is photocleavable to give a charged species for mass spectrometry.

36. The method of claim 35, wherein the ligand is an oligonucleotide.

37. The method of any one of claims 33 to 36, wherein the label is a group of formula $R^1R^2R^3X-$ or R^1R^2N-, where X is C or S, and R^1, R^2 and R^3 are the same or different and each is a monocyclic or fused ring aromatic group that is substituted or unsubstituted.

38. The method of claim 37, wherein at least one of R^1, R^2 and R^3 carries a substituent selected from C_1-C_{20} alkoxy or hydrocarbyl either unsubstituted or substituted by carboxylic acid, sulphonic acid, nitro, cyano, hydroxyl, thiol, primary, secondary or tertiary amino, primary, secondary or tertiary amido, anhydride, carbonyl halide or active ester.

39. A library of probes each comprising a ligand joined to a label by a link which is photocleavable to give a charged species for analysis by mass spectrometry, wherein each different probe has a different label.

40. The library of claim 39, wherein the ligand is an oligonucleotide.

41. The library of claim 39 or claim 40, wherein each label is a group of formula $R^1R^2R^3X-$ or R^1R^2N-, where X is C or S, and R^1, R^2 and R^3 are the same or different and each is a monocyclic or fused ring aromatic group that is substituted or unsubstituted.

42. The library of claim 41, wherein at least one of R^1, R^2 and R^3 carries a substituent selected from C_1-C_{20} alkoxy or hydrocarbyl either unsubstituted or substituted by carboxylic acid, sulphonic acid, nitro, cyano, hydroxyl, thiol, primary, secondary or tertiary amino, primary, secondary or

tertiary amido, anhydride, carbonyl halide or active ester.

43. A compound of formula $R^1R^2R^3XY$ or R^1R^2NY, where X is C or S, Y is a leaving group for reaction with a nucleophilic species, and R^1, R^2 and R^3 are the same or different and each is a monocyclic or fused ring aromatic group, at least one of which carries a substituent selected from C_1 – C_{20} alkoxy or hydrocarbyl either unsubstituted or substituted by carboxylic acid, sulphonic acid, nitro, cyano, hydroxyl, thiol, primary, secondary or tertiary amino, primary, secondary or tertiary amido, anhydride, carbonyl halide or active ester, provided that R^1, R^2 and R^3 together carry at least two amide groups and/or at least two N-hydroxysuccinimide ester groups.

FIGURE 1.

Coding Strategy using Trityl-based Mass-Tags.
1. Mass-tags are attached to oligos in solution

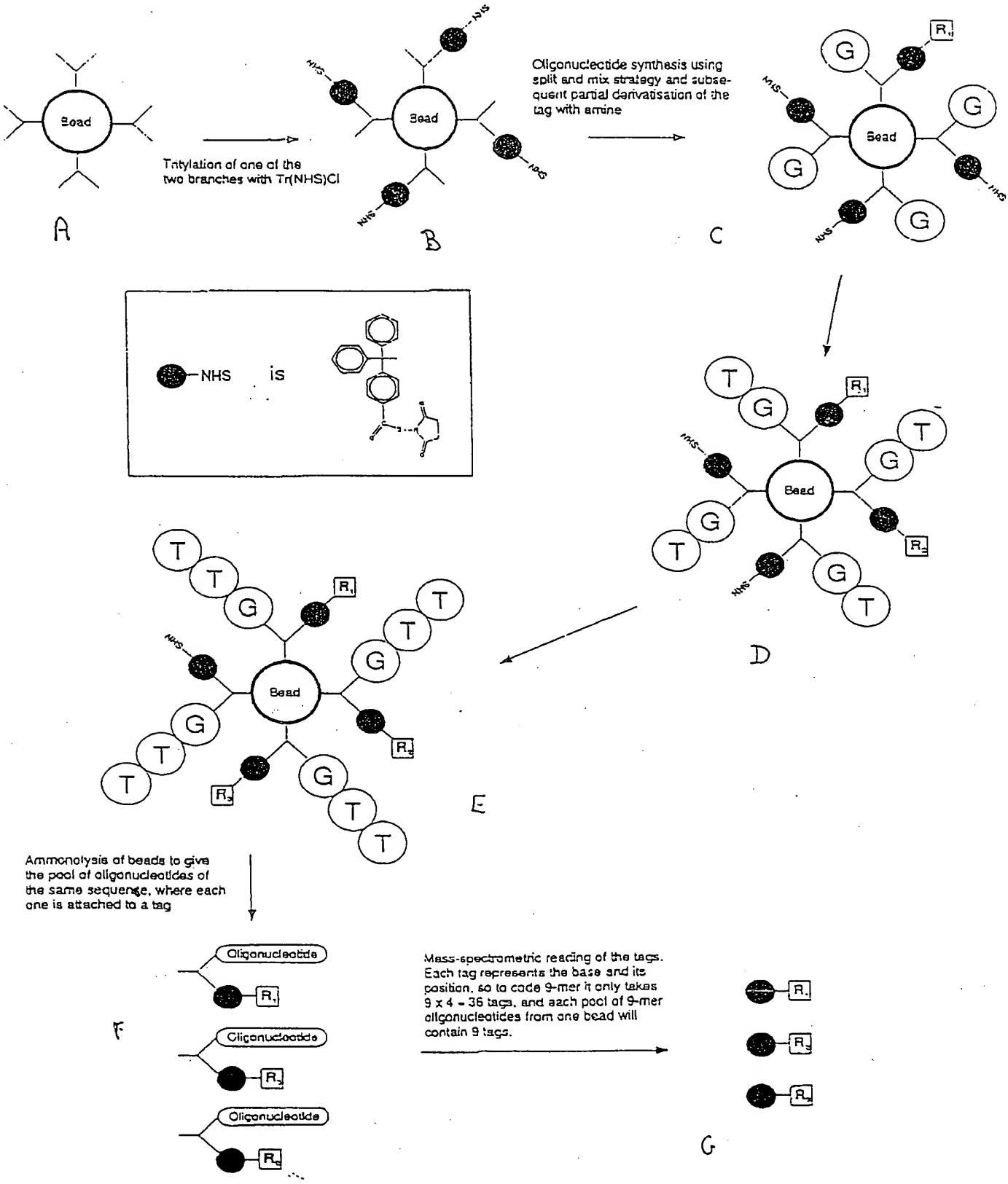


FIGURE 2

Coding Strategy using Trityl-based Mass-Tags.
2. Mass-tags and oligos attached to the bead

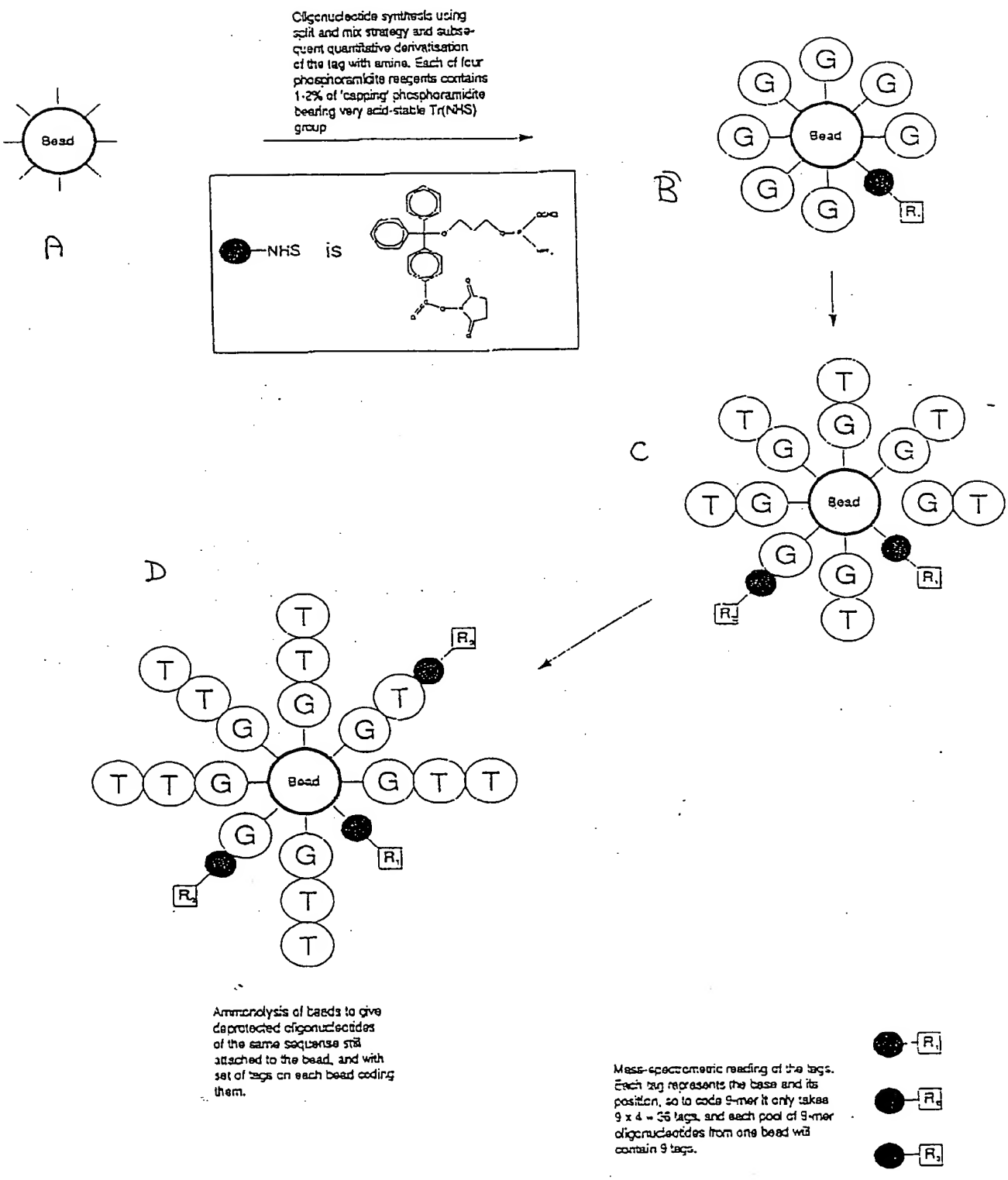


FIGURE 3

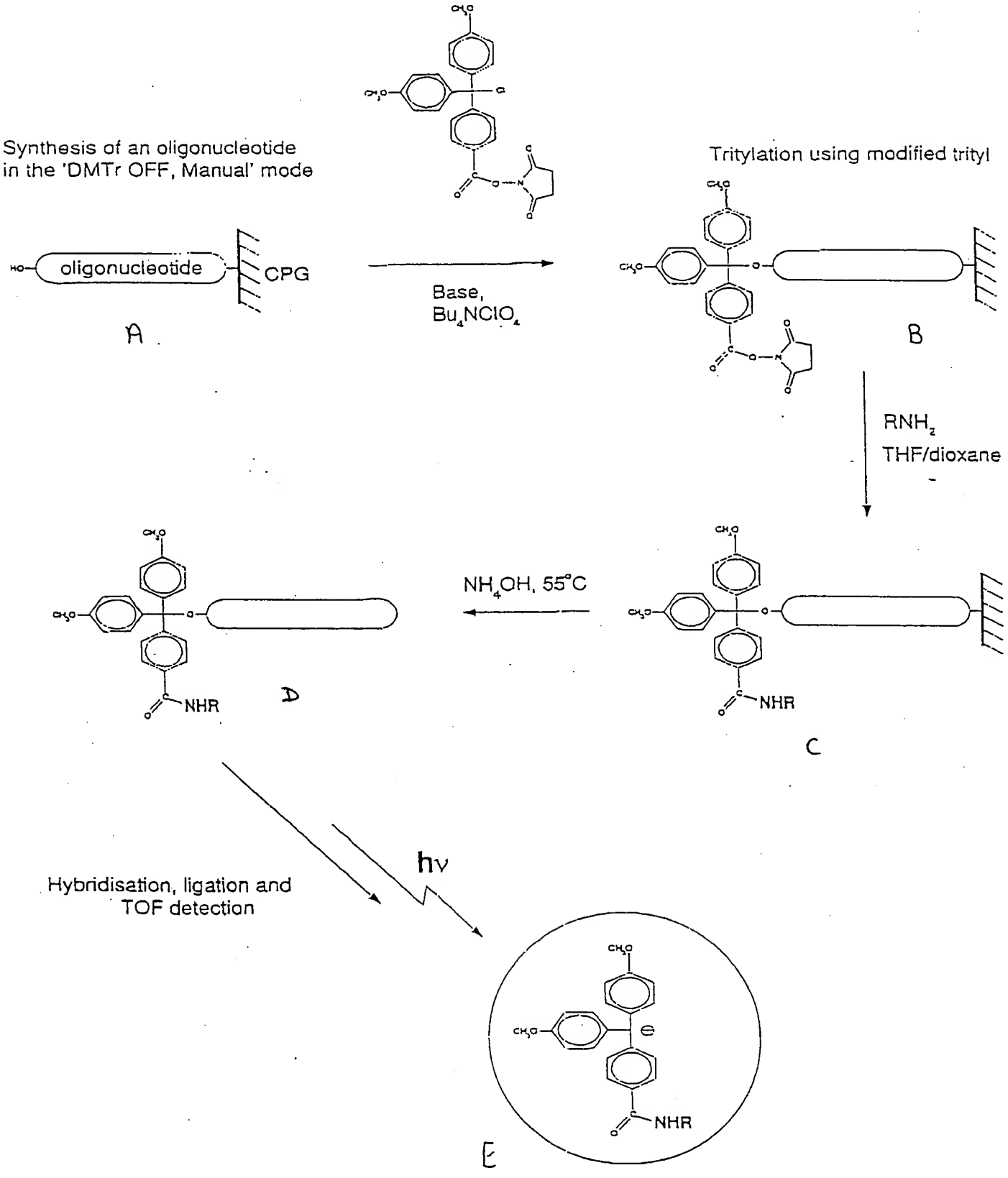

Synthesis of an oligonucleotide in the 'DMTr OFF, Manual' mode
Tritylation using modified trityl
oligonucleotide
CPG
A
Base,
Bu_4NClO_4
B
RNH_2
THF/dioxane
NH_4OH, 55°C
NHR
D
C
Hybridisation, ligation and TOF detection
hν
NHR
E

FIGURE 4

Synthesis of Trityl-Based Mass-Tags

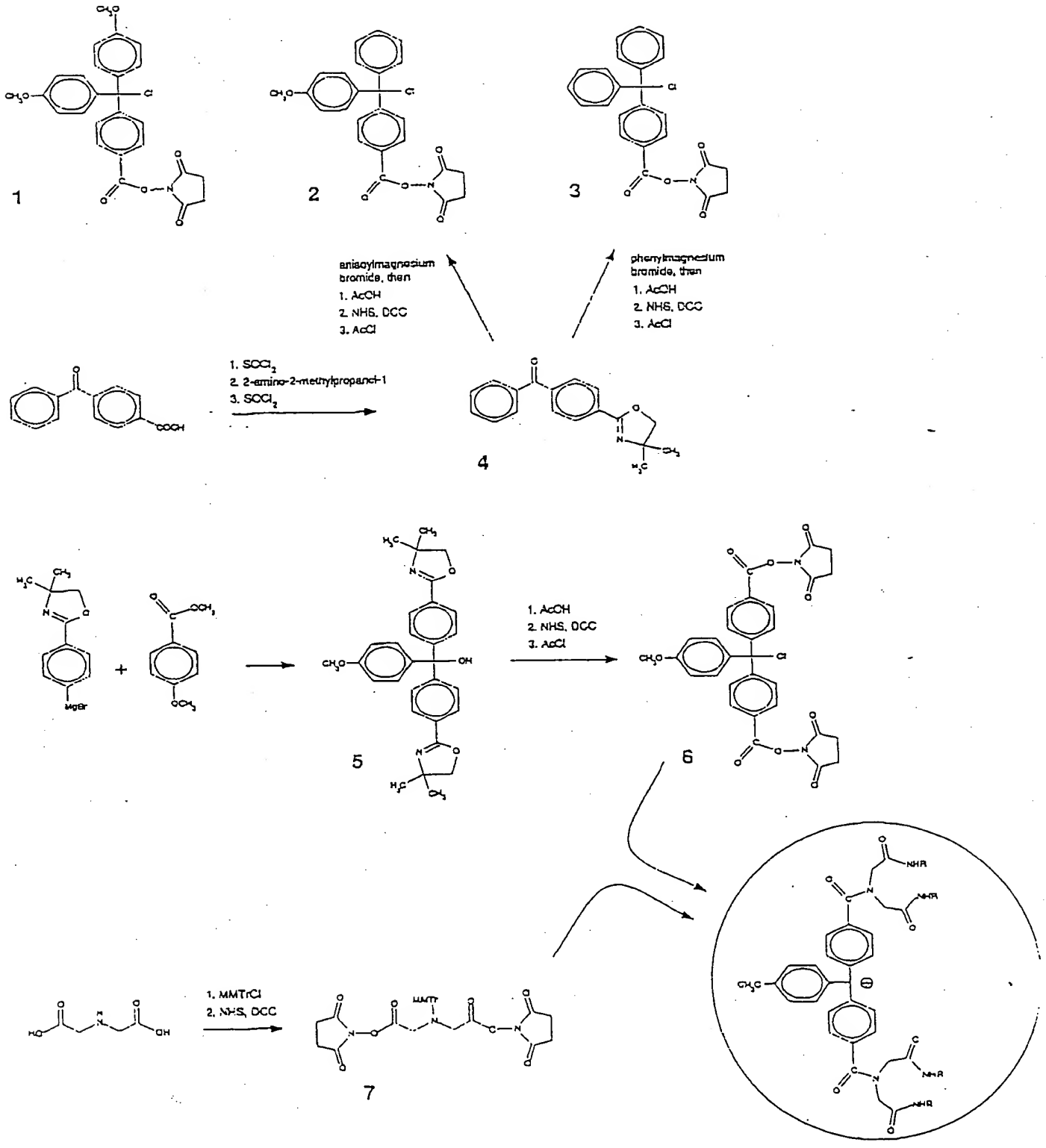
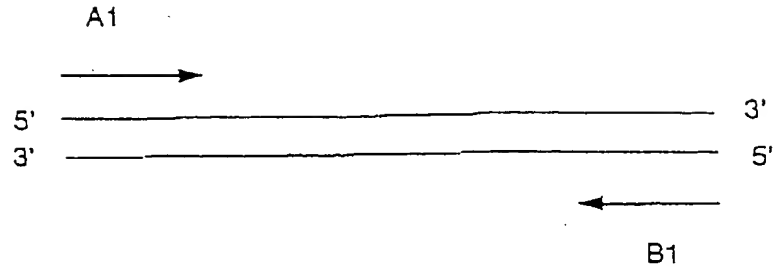
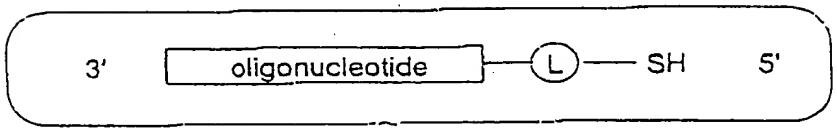


FIGURE 5

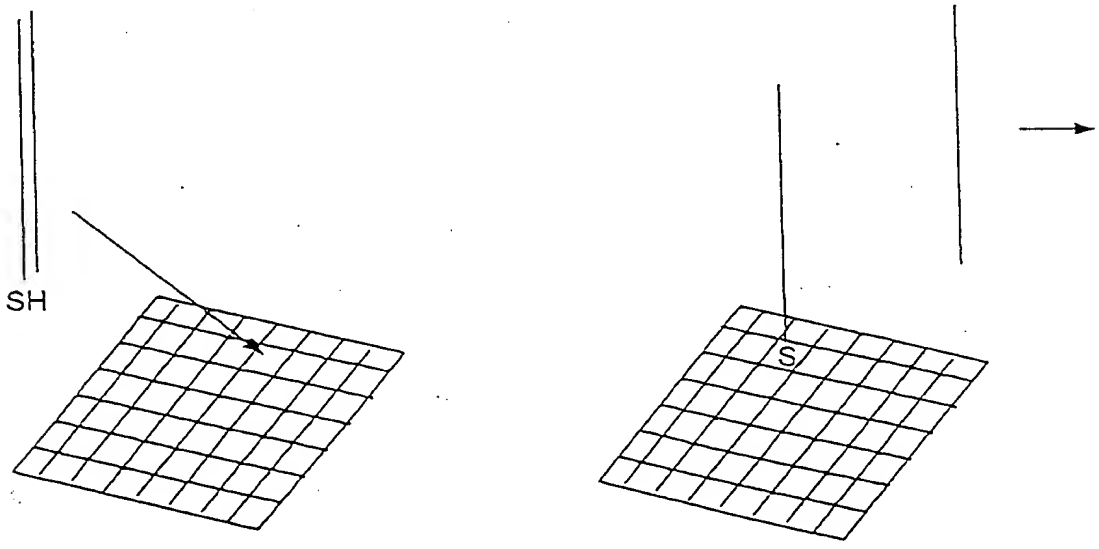
PCR AND IMMOBILISATION STRATEGY





ATTACHMENT

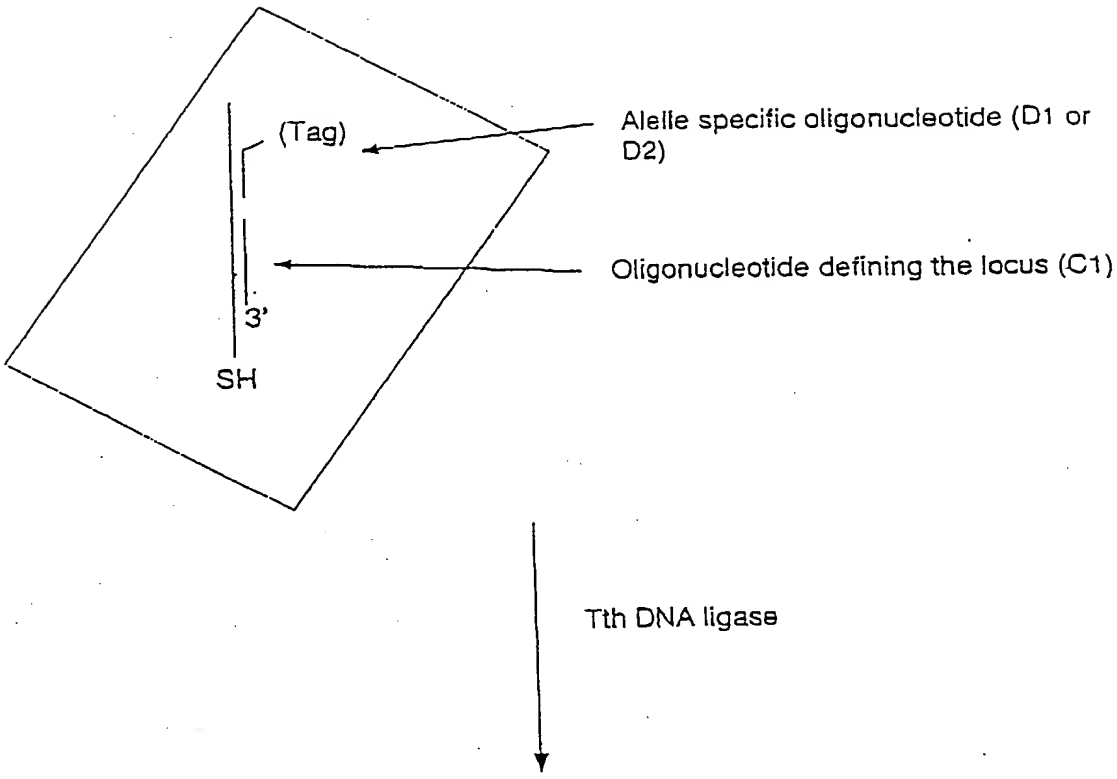
STRAND DISPLACEMENT



Gold Surface

FIGURE 6

HYBRIDISATION AND LIGATION



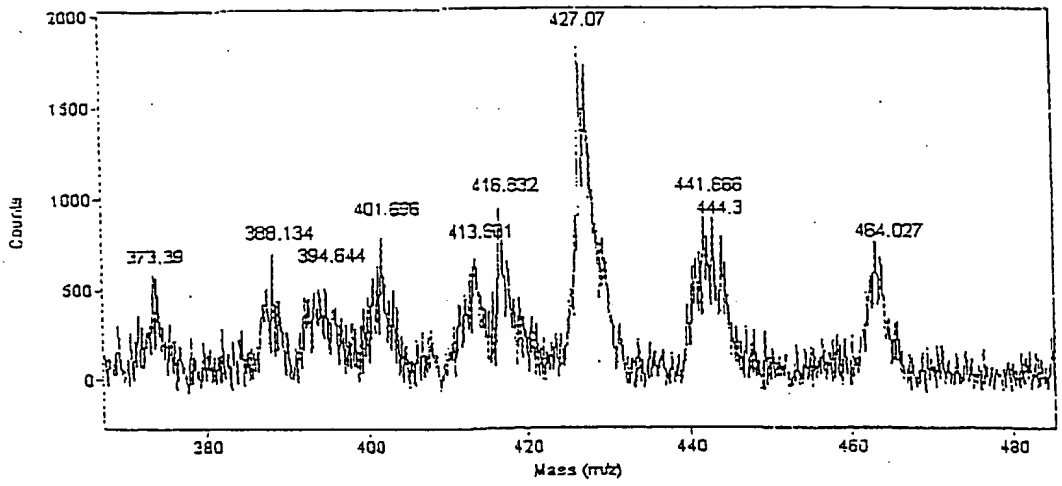

2000
1500
1000
500
0
Counts
427.07
416.832
441.866
444.3
401.656
413.581
388.134
394.644
373.39
464.027
380
400
420
440
460
480
Mass (m/z)

FIGURE 7

ABSTRACT

REAGENT AND METHOD

A method of making a set of labelled compounds by the use of a preferably particulate support, comprises dividing the support into lots, performing a different chemical reaction on each lot of the support, e.g. to couple a chemical moiety to that lot of the support, tagging a fraction of each lot of the support with a different label, and combining the said lots of the support. The steps are repeated several times, preferably to build up oligomer molecules carrying labels which identify the nature and position of a monomer unit of the oligomer, and which are releasable from the support. Preferred labels, which are releasable from the compounds by photocleavage to provide charged groups for analysis by mass spectrometry, are groups of the trityl (trimethylphenyl) family. Also claimed are libraries of these labels and their use in assays and sequencing methods.